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Esterase-Like Activity of Human Serum Albumin: Structure-Activity Relationships for the Reactions with Phenyl Acetates and *p*-Nitrophenyl Esters[1]

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In order to investigate the reactivity of human serum albumin (HSA) with ester-type drugs and to characterize the site of the esterase-like activity, the Michaelis constants (Ks) and the catalytic rate constants (k_{cat}) were determined for the reactions of phenyl acetates and *p*-nitrophenyl esters with HSA at 25°. A linear relationship between log k_{cat} and Hammett σ^- values was found for phenyl acetates at pH 9.9; its slope was +1.52. It is suggested that aspirin also reacts with HSA by the same mechanism. The effects of aromatic substituents on the K_s values were small. The K_s values for *p*-nitrophenyl esters at pH 7.0 were correlated with Hansch's π and Taft's E_s values as follows; log $K_s = -0.578\,\pi - 0.184\,E_s - 3.566$ ($r = 0.963$). The hydrophobic interaction was predominant in the binding of the substrates to HSA. The log k_{cat}—pH profile obtained for *p*-nitrophenyl acetate indicates the participation of a single catalytic group, p$K_a \fallingdotseq 9.5$, in this reaction.

Keywords——human serum albumin; esterase-like activity of human serum albumin; structure-activity relationship; regression analysis; hydrophobic interaction; reactive site of human serum albumin; enzyme kinetics; reaction of aspirin with human serum albumin; phenyl acetates; *p*-nitrophenyl esters

In the previous paper[3] it was reported that the reaction rate of *p*-nitrophenyl acetate (**6**) with human serum albumin (HSA) decreased in the presence of drugs such as N-arylanthranilic acids, coumarin derivatives and prostaglandins, and these inhibitions were analyzed kinetically. The kinetic method is useful for studies on drug-HSA interactions, since information relating to the drug binding sites and to the binding affinity of the drugs for the reactive site of HSA can be obtained. Recently, Koh and Means[4] have studied the interaction of small fatty acid anions with HSA by a similar kinetic method, and suggested that the anion binding site appeared to be a relatively small, uniform apolar cavity with one or more cationic groups located near one end. It is important not only to characterize the drug binding site of HSA but also to investigate the reactivity of HSA with the esters, since HSA may affect the cleavage of ester-type drugs *in vivo*.

To elucidate the reactivity of HSA with the ester drugs and to characterize the reactive site, the reactions of phenyl acetates and *p*-nitrophenyl esters with HSA were investigated kinetically in this study. The Michaelis constants and catalytic rate constants for the reactions were determined. The relationships between the structure of the substrates and the reactivity of HSA were examined.

Experimental

Materials——HSA (Sigma Chem. Co., Fraction V, lot 47c-04421; the same lot number as that employed in the previous study[3]) was used after purification by Chen's method.[5] The molecular weight of HSA was

1) Presented at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, Japan, August 1979.
2) Location: *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) K. Ikeda, Y. Kurono, Y. Ozeki, and T. Yotsuyanagi, *Chem. Pharm. Bull.* (Tokyo), **27**, 80 (1979).
4) S.M. Koh and G.E. Means, *Arch. Biochem. Biophys.*, **192**, 73 (1979).
5) R.F. Chen, *J. Biol. Chem.*, **242**, 173 (1967).

Table I. Substrates Used, and Melting or Boiling Points of Substrates synthesized by the Method of Spasov

	Substrates	mp or bp (°C) (°C/mmHg)	Literature values mp or bp (°C) (°C/mmHg)
1	aspirin		
2	*p*-methoxyphenyl acetate	32—33	31—32[8]
3	*p*-chlorophenyl acetate	76—80/4	108/12.5[9a]
4	*m*-chlorophenyl acetate	72—73/2	116.5/21[9b]
5	*m*-nitrophenyl acetate	53—55	55—56[10]
6	*p*-nitrophenyl acetate		
7	*p*-nitrophenyl propionate		
8	*p*-nitrophenyl butyrate		
9	*p*-nitrophenyl valerate		
10	*p*-nitrophenyl capronate		
11	*p*-nitrophenyl iso-butyrate	37—38	36.5—37[11]
12	*p*-nitrophenyl iso-valerate	123/1.5	158—160/6[11]
13	*p*-nitrophenyl trimethylacetate	93—95	94—95[11]

assumed to be 69000 and the concentration was determined based on an extinction coefficient $E_{278}^{0.1\%}$ of 0.531 at 278 nm.[6] The substrates used for HSA are listed in Table I; the melting or boiling points for the substrates synthesized by the method of Spasov[7] are listed, together with the literature values.[8–11] All other chemicals used were obtained commercially and were of reagent grade. The numbers in Table I are those used here to refer to the substrates.

Procedures for Kinetic Runs——The reaction of the substrate with HSA was carried out in the presence of excess HSA to avoid complications due to the multiple reactive sites of HSA,[6] as described in the previous paper.[3] The reaction rate was followed in terms of the ultraviolet (UV) spectral changes due to the appearance of the corresponding phenol. An experimentally convenient wavelength was employed. The pseudo first order rate constant was determined from the plot of log ($Ab_\infty - Ab$) *versus* time, where Ab_∞ and Ab are the absorbances at completion of the reaction and at time *t*, respectively. The temperature was 25°.

Sörensen buffer (1/15 M phosphate for pH 6.0 to 8.0 and 1/20 M borate for pH 8.0 to 10.5) were used to investigate the pH profiles of the reaction parameters of **6** with HSA. The ionic strength of each buffer was not fixed, because the addition of NaCl to the buffer decreased the reaction rates in a complicated manner. For the fast reaction of **6** with HSA in the alkaline region, a stopped flow apparatus (Union Giken RA-1100) was employed. In the neutral region, a Hitachi 124 UV spectrophotometer equipped with a thermostated cell was used. The initial concentration of **6** was about 2.0×10^{-5} M and the HSA concentration was varied from about 7.0×10^{-5} to 4.0×10^{-4} M.

The reactions of phenyl acetates with HSA were carried out in 2/5 M carbonate buffer, pH 9.9, since the phenolate anions released had larger molar absorptivities (ε) than the free phenols. The initial concentrations of phenyl acetates were about 1.0×10^{-4} M, depending on the ε values of the phenolate anions. The initial HSA concentrations corresponded to a three to six-fold excess relative to those of the substrates.

The reactions of *p*-nitrophenyl esters with HSA were followed in pH 7.0 phosphate buffer. The initial concentrations of the substrates were about 3.0×10^{-6} M and those of HSA were from 1.5×10^{-5} to 1.0×10^{-4} M.

Determination of Reaction Parameters——On the basis of the study by Means and Bender[6] and our previous results,[3] the reactions of the substrates with HSA were assumed to proceed through the pathway

$$\begin{array}{ccccc} \text{EST} + \text{HSA} & \overset{K_S}{\rightleftharpoons} & \text{EST}\cdot\text{HSA} & \xrightarrow{k_{cat}} & \text{phenol} + \text{Acyl–HSA} \\ \downarrow k_0 & & & & \downarrow k_{deacyl} \\ \text{phenol} + \text{acid} & & & & \text{acid} + \text{HSA} \end{array}$$

Chart 1

6) G.E. Means and M.L. Bender, *Biochemistry*, **14**, 4989 (1975).
7) A. Spasov, *Chem. Abstr.*, **34**, 2343 (1940).
8) Beilsteins Handbuch, Ersters Erganzungswerk, Bd. VI, p. 416.
9) *a*) Beilsteins Handbuch, Hauptwerk, Bd. VI, p. 187; *b*) *ibid.*, p. 185.
10) F. Arnall, *J. Chem. Soc.*, **125**, 814 (1921).
11) S. Kreisky, *Acta Chem. Scand.*, **11**, 913 (1957).

shown in Chart 1. In this chart, EST is the substrate and EST·HSA is the Michaelis-Menten type complex between EST and HSA. Acyl-HSA is HSA acylated by the substrate. K_S denotes the dissociation constant of the complex. The rate constants of EST·HSA and EST are represented by k_{cat} and k_0, respectively. k_{deacyl} is the rate constant of Acyl-HSA.

To determine the effect of k_{deacyl} on the apparent rate of phenol release, acetic acid formation rates in the reaction of **6** with HSA at pH 7.0 and 10.0 were preliminarily measured by means of a pH-stat apparatus (Toa-Dempa HS-20A). The rates of acetic acid formation were very small compared with those of the *p*-nitrophenol release. Thus, the influence of the deacylation rate on the observed rate of phenol appearance could be neglected under the experimental conditions employed.

The pseudo first order rate constant of phenol release, k_{obs}, can be represented as follows.

$$k_{obs} = \frac{k_0 K_S + k_{cat}[HSA]}{K_S + [HSA]} \quad (1)$$

Equation (1) can be rearranged to give the following equation (2), which is the same as that in the previous report.[3]

$$\frac{1}{k_{obs} - k_0} = \frac{K_S}{(k_{cat} - k_0)[HSA]_0} + \frac{1}{k_{cat} - k_0} \quad (2)$$

Here, the concentration of HSA in equation (1) is approximated to $[HSA]_0$, the initial concentration of HSA, because the concentration of HSA is much higher than that of the substrate. A linear relationship between $1/(k_{obs} - k_0)$ and $1/[HSA]_0$ was obtained in all cases. The K_S value was obtained from the slope divided by the intercept, and the value of k_{cat} was calculated from the intercept and k_0.

Results and Discussion

The pH Profiles of the Reaction Parameters of *p*-Nitrophenyl Acetate with HSA

Means and Bender presented a pH-rate profile for the reaction of **6** with HSA and suggested the participation of a basic group with a pK_a value near 8.7 in the reaction.[6] The second-order rate constants in their report, however, seem to be values of k_{cat}/K_S (M^{-1} sec^{-1}). In contrast, the pH dependencies of the individual kinetic parameters, *i.e.* K_S and k_{cat}, were separately examined in the present work.

Figure 1 shows the pH profiles of K_S and k_{cat} for the reaction of **6** with HSA. The K_S values are slightly dependent on pH over the range from 6 to 10. This indicates that the binding is little affected by pH changes. The value of K_S at pH about 10.5 was larger than that in the neutral region. This large K_S value may be related to the effect of an ionic group(s) near the reactive site of HSA on the binding, or to the slight conformation change of HSA above pH 10.[12a]

The values of k_{cat} were markedly dependent on pH. The slope of the log k_{cat}-pH profile between pH 6 and 9 was about unity, and over pH 9 a plateau gradually appeared with increasing pH. This profile suggests the involvement of a single catalytic group in HSA for the reaction, as in the mechanism proposed by Means and Bender.[6] The pK_a value of the catalytic group appears to be around 9.5, which is rather different from the value, 8.7, presented by Means and Bender. This discrepancy in the pK_a values might arise from the different pH profiles, that is, log (k_{cat}/K_S) *versus* pH and log k_{cat} *versus* pH.

Structure-Activity Relationships for the Reaction of Phenyl Acetates with HSA

The rates and dissociation constants for the reactions of phenyl acetates with HSA are listed in Table II. Table II also shows the spontaneous rate constants (k_0) of the substrates, the pK_a values of the corresponding phenols[13] and the Hammett σ^- values.[14]

12) *a*) V.M. Rosenor, M. Oratz, and M.A. Rothschild, "Albumin Structure, Function and Uses," ed. by J.F. Foster, Pergamon Press, Oxford, London, 1977, p. 78; *b*) *idem*, ed. by J.R. Brown, p. 27; *c*) *idem*, ed. by R.P. Taylor, p. 183.
13) G. Kortüm, W. Vogel, and K. Andrussow, "Dissociation Constants of Organic Acids in Aqueous Solution," Butterworths, London, 1961.
14) H.H. Jaffé, *Chem. Rev.*, 53, 191 (1953).

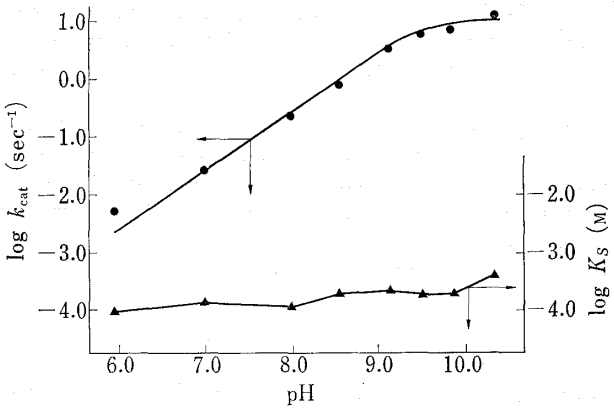

Fig. 1. The pH Profiles of Rate and Dissociation Constants for the Reaction of *p*-Nitrophenyl Acetate with HSA at 25°

●: log k_{cat} *vs.* pH.
▲: log K_S *vs.* pH.

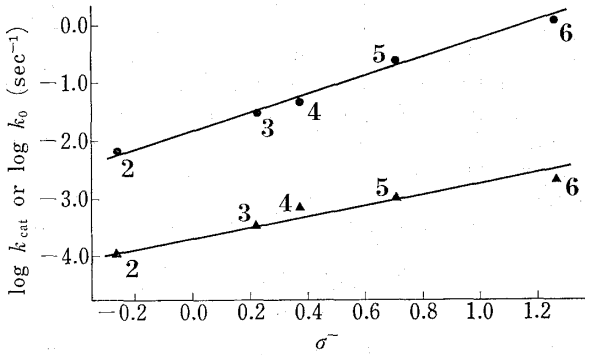

Fig. 2. Plots of Rate Constants *versus* Hammett σ^- Values

●: log k_{cat} *vs.* σ^-.
▲: log k_0 *vs.* σ^-.
Numbers refer to those in Table I.

Table II. Rate and Dissociation Constants for the Reactions of Phenyl Acetates in the Presence and Absence of HSA[a]

Substrates[b]	k_{cat} (sec^{-1})	K_S (M)	k_0 (sec^{-1})	pK_a[c]	σ^-[d]
1	4.00×10^{-4}	2.17×10^{-3}	2.10×10^{-5}	12.38	—
2	7.30×10^{-3}	6.59×10^{-4}	1.23×10^{-4}	10.20	−0.268
3	2.97×10^{-2}	5.40×10^{-4}	3.87×10^{-4}	9.42	0.227
4	4.42×10^{-2}	6.43×10^{-4}	7.22×10^{-4}	9.08	0.372
5	2.65×10^{-1}	4.60×10^{-4}	1.18×10^{-3}	8.39	0.710
6	1.24	3.75×10^{-4}	2.25×10^{-3}	7.16	1.270

a) Obtained at pH 9.9 and 25°.
b) Numbers refer to those in Table I.
c) pK_a of the corresponding phenols.[13]
d) Hammett σ^- values.[14]

Figure 2 shows the relationships between the rate constants and the Hammett σ^- values. For both k_{cat} and k_0 values, good linear relationships were obtained. A plot of log k_{cat} *versus* σ^- gave a slope of +1.52. The positive value of the slope suggests that the reaction of the substrate with HSA proceeds through nucleophilic attack of the catalytic group of HSA to the carbonyl carbon atom of the substrate. The finding that the slope is steeper than that for the spontaneous reaction (k_0) indicates that the reaction occurs in a nonpolar region of HSA. The slope of the log k_0 *versus* σ^- plots was +0.82, for comparison.

To correlate the result for **1** with those for the other substrates in Table II, the rate constants were plotted against the pK_a values of the corresponding phenols. Here again, there were good linear relationships, as shown in Fig. 3. The correlation implies that the same reactive site of HSA participates in the reaction for all the substrates including **1**, since an approximately linear relationship between the pK_a values of the phenols and Hammett σ^- values was found.[15]

Hawkins and his co-workers[16] reported that the lysine residue in HSA was acetylated by aspirin *in vivo* and *in vitro*, although kinetic studies were not carried out. The position of this residue was shown to be lysine-199 according to Brown's completed sequence of

15) J. Hine, "Physical Organic Chemistry," 2nd Ed., McGraw-Hill Inc., New York, N.Y., 1962, p. 89.
16) *a*) D. Hawkins, R.N. Pinkard, and R.S. Farr, *Science*, **160**, 780 (1968); *b*) D. Hawkins, R.N. Pinkard, I.P. Crawford, and R.S. Farr, *J. Clin. Inves.*, **48**, 536 (1969); *c*) R.N. Pinkard, D. Hawkins, and R.S. Farr, *Ann. N.Y. Acad. Sci.*, **266**, 341 (1974).

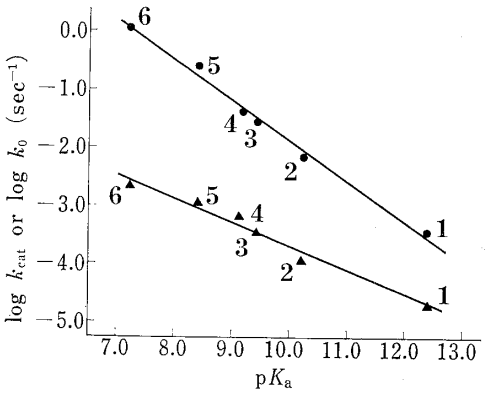

Fig. 3. Plots of Rate Constants *versus* pK_a Values of Corresponding Phenols

●: log k_{cat} *vs.* pK_a.
▲: log k_0 *vs.* pK_a.
Numbers refer to those in Table I.

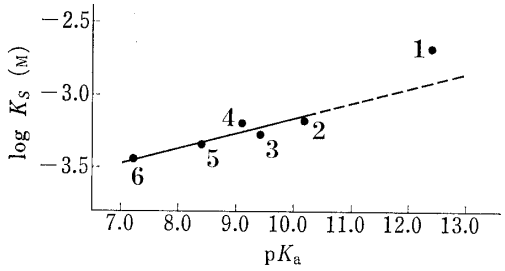

Fig. 4. Relationship between Dissociation Constants for Phenyl Acetates and pK_a Values of Corresponding Phenols

Numbers refer to those in Table I.

HSA.[12b,17,18] As shown in Fig. 3, aspirin reacted with HSA by the same mechanism as with other substrates, so it is possible that the catalytic group is the free (unprotonated) ε-amino group of lysine-199 for the substrates **1**—**6**. However, the reaction rates of **6** with HSA acetylated at pH 7.3 and 10.0 according to the method of Hawkins *et al.*[16] did not significantly alter. This result appears to rule out the lysine residue. Another possible candidate is the hydroxyl group of tyrosine,[12b,c] as proposed by Tildon and Ogilvie for the reaction of **6** with bovine mercaptalbumin.[19] Identification of the reactive group of HSA for these substrates is in progress.

Figure 4 illustrates the relationship between log K_S and pK_a of the corresponding phenol. A slight positive correlation between log K_S and pK_a was observed; the deviation of aspirin from the line may be attributed to the carboxyl group at the *ortho*-position. Thus, the difference between the log K_S values for **2** and **6**, *i.e.* the maximum and the minimum, respectively, in Table II except for aspirin, was only 0.242 log unit. Accordingly, the effects of aromatic substituents on substrate binding to HSA appears to be small.

Table III. Dissociation and Rate Constants for the Reactions of *p*-Nitrophenyl Esters with HSA[a]

Substrates[b]	K_S (M)	k_{cat} (sec^{-1})	π[c]	E_s[d]	σ^* [e]
6	1.55×10^{-4}	2.93×10^{-2}	0.50	0.00	0.00
7	8.69×10^{-5}	7.15×10^{-2}	1.00	−0.07	−0.10
8	4.29×10^{-5}	1.02×10^{-2}	1.50	−0.36	−0.12
9	2.57×10^{-5}	2.58×10^{-3}	2.00	−0.39	−0.13
10	1.11×10^{-5}	2.22×10^{-3}	2.50	−0.40	−0.16
11	3.59×10^{-5}	1.08×10^{-2}	1.30	−0.47	−0.19
12	3.85×10^{-5}	1.47×10^{-3}	1.80	−0.93	−0.13
13	4.11×10^{-5}	6.42×10^{-4}	1.98	−1.54	−0.30

a) Obtained at pH 7.0 and 25°.
b) Numbers refer to those in Table I.
c) Hansch's hydrophobic substituent constant.[20,22]
d) Taft's steric substituent constant.[21,22]
e) Taft's polar substituent constant.[21,22]

17) J.R. Brown, *Fed. Proc.*, **34**, 591 (1975).
18) K.K. Gambhir, R.H. McMenamy, and F. Watson, *J. Biol. Chem.*, **250**, 6711 (1975).
19) J.T. Tildon and J.W. Ogilvie, *J. Biol. Chem.*, **247**, 1265 (1972).

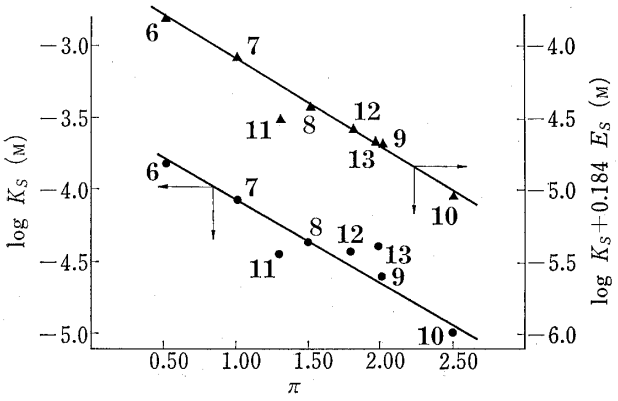

Fig. 5. Plots of log K_S *versus* π, and of log K_S $+0.186\ E_s$ *versus* π for *p*-Nitrophenyl Esters

●: log K_S *vs.* π.
▲: log K_S+0.186 E_s *vs.* π.
Numbers refer to those in Table I.

Structure-Activity Relationships for Reactions of *p*-Nitrophenyl Esters with HSA

Table III lists the kinetic parameters for the reactions of *p*-nitrophenyl esters with HSA. The dissociation and rate constants varied widely. Regression analyses in studies of enzymic reactions, for example α-chymotrypsin, using substituent constants such as Hansch's π-value[20] and Taft's E_S- and σ^*-values,[21] have been carried out by Hansch and his co-workers,[22,23] and Milstien and Fife.[24] The values of π, E_S and σ^* were used as measures of hydrophobicity, steric effect and polarity of the substrate, respectively; these are listed in Table III for convenience. Similar regression analyses were attempted for the K_S and k_{cat} values listed in Table III.

The plot of log K_S against π-value is shown in Fig. 5. A fairly good relationship was obtained and its regression line can be expressed as

$$\log K_S = -0.501(\pm 0.189)\pi - 3.591(\pm 0.318) \qquad (3)$$
$$n=8,\ s=0.130,\ r=0.935$$

where the values in parentheses are the 95% confidence intervals; n, s and r represent the number of compounds employed, the standard deviation and the correlation coefficient, respectively. Since the points of **11**, **12** and **13** (which have side chains) departed slightly from the line, a steric factor E_S was added to the analysis. The results obtained by the least-squares method can be summarized as

$$\log K_S = -0.578(\pm 0.196)\pi - 0.184(\pm 0.250)E_S - 3.566(\pm 0.281) \qquad (4)$$
$$n=8,\ s=0.108,\ r=0.963$$

Figure 5 also shows a plot of (log K_S+0.184 E_S) *versus* π. Although the E_S term in equation (4) was not statistically significant at the 0.95 level of significance, the points for **12** and **13** appear to be better fitted than in the case of equation (3). The effects of aromatic substituents on the K_S values were small (Fig. 4), while the binding of *p*-nitrophenyl esters to the reactive site of HSA was markedly affected by substituents at the acyl portion. These results suggest that the alkyl chains of the acyl group are mainly bound to the HSA reactive site by hydrophobic forces.

We further attempted to correlate the values of k_{cat} in Table III with various factors such as Taft's σ^* and E_S values, *etc.*, but without success. Thus, other factors, so far unidentified, may affect the k_{cat} values in the reactions of *p*-nitrophenyl esters with HSA.

20) T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, **86**, 5175 (1964).
21) R.W. Taft, "Steric Effects in Organic Chemistry," ed. by M.S. Newman, John Willey and Sons, Inc., New York, N.Y., 1956, p. 644.
22) C. Hansch and E. Coats, *J. Pharm. Sci.*, **59**, 731 (1970).
23) C. Hansch, E.W. Deutsch, and R.N. Smith, *J. Am. Chem. Soc.*, **87**, 2738 (1965).
24) J.B. Milstien and T.H. Fife, *Biochemistry*, **8**, 623 (1969).